AMERICAN SOCIETY FOR MICROBIOLOGY | Infection and Immunity®

MICROBIAL IMMUNITY AND VACCINES

Antibodies to Intercellular Adhesion Molecule 1-Binding *Plasmodium falciparum* Erythrocyte Membrane Protein 1-DBLβ Are Biomarkers of Protective Immunity to Malaria in a Cohort of Young Children from Papua New Guinea

Sofonias K. Tessema,[a,b] Digjaya Utama,[a,b] Olga Chesnokov,[c] Anthony N. Hodder,[a,b] Clara S. Lin,[a,b] G. L. Abby Harrison,[a,b] Jakob S. Jespersen,[d,k] Bent Petersen,[e,f] Livingstone Tavul,[g] Peter Siba,[g] Dominic Kwiatkowski,[h,i] Thomas Lavstsen,[d,k] Diana S. Hansen,[a,b] Andrew V. Oleinikov,[c] Ivo Mueller,[a,b,j] Alyssa E. Barry[a,b]

[a]The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia

[b]The University of Melbourne, Department of Medical Biology, Victoria, Australia

[c]Charles E. Schmidt College of Medicine, Florida Atlantic University, Boca Raton, Florida, USA

[d]Centre for Medical Parasitology, Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark

[e]Center for Biological Sequence Analysis, Technical University of Denmark, Kgs. Lyngby, Denmark

[f]Centre of Excellence for Omics-Driven Computational Biodiscovery (COMBio), Faculty of Applied Sciences, AIMST University, Kedah, Malaysia

[g]Vector Borne Diseases Unit, Papua New Guinea Institute of Medical Research, Goroka, Papua New Guinea

[h]Wellcome Trust Sanger Institute, Hinxton, United Kingdom

[i]MRC Centre for Genomics and Global Health, University of Oxford, Oxford, United Kingdom

[j]Institut Pasteur, Paris, France

[k]Department of Infectious Diseases, Copenhagen University Hospital, Copenhagen, Denmark

ABSTRACT *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) mediates parasite sequestration to the cerebral microvasculature via binding of DBLβ domains to intercellular adhesion molecule 1 (ICAM1) and is associated with severe cerebral malaria. In a cohort of 187 young children from Papua New Guinea (PNG), we examined baseline levels of antibody to the ICAM1-binding PfEMP1 domain, DBL$\beta3_{PF11_0521}$, in comparison to four control antigens, including NTS-DBLα and CIDR1 domains from another group A variant and a group B/C variant. Antibody levels for the group A antigens were strongly associated with age and exposure. Antibody responses to DBL$\beta3_{PF11_0521}$ were associated with a 37% reduced risk of high-density clinical malaria in the follow-up period (adjusted incidence risk ratio [aIRR] = 0.63 [95% confidence interval {CI}, 0.45 to 0.88; $P = 0.007$]) and a 25% reduction in risk of low-density clinical malaria (aIRR = 0.75 [95% CI, 0.55 to 1.01; $P = 0.06$]), while there was no such association for other variants. Children who experienced severe malaria also had significantly lower levels of antibody to DBL$\beta3_{PF11_0521}$ and the other group A domains than those that experienced nonsevere malaria. Furthermore, a subset of PNG DBLβ sequences had ICAM1-binding motifs, formed a distinct phylogenetic cluster, and were similar to sequences from other areas of endemicity. PfEMP1 variants associated with these DBLβ domains were enriched for DC4 and DC13 head structures implicated in endothelial protein C receptor (EPCR) binding and severe malaria, suggesting conservation of dual binding specificities. These results provide further support for the development of specific classes of PfEMP1 as vaccine candidates and as biomarkers for protective immunity against clinical *P. falciparum* malaria.

KEYWORDS DBLβ, EPCR, ICAM1, Papua New Guinea, PfEMP1, antibodies, diversity, malaria, *var* genes

Received 10 August 2017 **Returned for modification** 3 October 2017 **Accepted** 18 May 2018

Accepted manuscript posted online 21 May 2018

Citation Tessema SK, Utama D, Chesnokov O, Hodder AN, Lin CS, Harrison GLA, Jespersen JS, Petersen B, Tavul L, Siba P, Kwiatkowski D, Lavstsen T, Hansen DS, Oleinikov AV, Mueller I, Barry AE. 2018. Antibodies to intercellular adhesion molecule 1-binding *Plasmodium falciparum* erythrocyte membrane protein 1-DBLβ are biomarkers of protective immunity to malaria in a cohort of young children from Papua New Guinea. Infect Immun 86:e00485-17. https://doi.org/10.1128/IAI.00485-17.

Editor John H. Adams, University of South Florida

Address correspondence to Alyssa E. Barry, barry@wehi.edu.au.

Malaria due to infection with *Plasmodium falciparum* remains a major global public health issue, with more than 400,000 deaths and 215 million symptomatic episodes each year (1). Children with limited prior exposure to malaria bear the majority of the disease burden; however, naturally acquired immunity eventually develops with age and exposure and is associated with the acquisition of a diverse repertoire of antibodies to parasite-encoded variant antigens on the infected erythrocyte surface (2). The major target of this immunity is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (3, 4), which is differentially encoded by up to 60 highly polymorphic *var* genes per parasite genome (5–7). Expression of diverse PfEMP1/*var* gene variants allows clonal antigenic variation (8, 9) and cytoadhesion to a wide variety of host molecules, including chondroitin sulfate A (10), CD36 (11), endothelial protein C receptor (EPCR) (12), and intercellular cytoadhesion molecule 1 (ICAM1) (13). Adhesion occurs via specialized PfEMP1 domains, known as Duffy binding like (DBL) and cysteine-rich interdomain region (CIDR) (7). While antibodies to PfEMP1 in general have been shown to be important mediators of protection against symptomatic malaria, the specific PfEMP1 variants targeted by protective immune responses are poorly understood.

var genes have been classified into three major groups (A, B, and C) based on chromosome orientation and conserved structural and sequence features, and in addition, there exists a group of chimeric genes (B/A), also known as domain cassette 8 (DC8) (14). Group A and B/A *var* genes are expressed in parasites isolated from children with severe disease and are upregulated in cytoadherent parasites linked to pathogenesis (reviewed in references 15 and 16). PF11_0521 and PFD1235w are group A *var* genes that contain ICAM1-binding DBLβ domains (17–19). Infected erythrocytes colocalize with ICAM1 expression in the brain blood vessels, suggesting that ICAM1 mediates parasite sequestration in cerebral malaria (20). These genes also belong to the subclass of group A PfEMP1 variants that have adjacent CIDR domains that bind EPCR (21), another important host-parasite interaction implicated in severe malaria (12). Dual binding to these host receptors has been linked to cerebral malaria (21). To our knowledge, only one study, conducted in Tanzanian children, has found an association between high levels of antibodies against the ICAM1-binding DBLβ domain of PF11_0521 (DBL$\beta3_{PF11_0521}$) and a reduced risk of severe malaria (22). The role of antibodies against ICAM1-binding DBLβ in protection against clinical and severe malaria is thus not well understood and has not been studied outside sub-Saharan Africa.

Considering the diverse functional roles of different PfEMP1 variants and domains, protective immunity would be expected to vary considerably among different PfEMP1 subgroups and cytoadherent domains. Therefore, this study aimed to investigate whether antibodies against DBL$\beta3_{PF11_0521}$ are associated with protection against clinical and severe malaria in comparison to domains from other PfEMP1 variants not associated with ICAM1 binding or severe malaria (23, 24). The study was conducted in a longitudinal cohort of very young (age, 1 to 3 years) children from Papua New Guinea (PNG) who were actively acquiring immunity to malaria (25) to minimize the background of diverse PfEMP1 antibodies that are acquired with high malaria exposure (25–27). Plasma antibody levels were measured at baseline and associated with a prospective risk of uncomplicated (clinical) and severe malaria. To explore the PfEMP1 landscape of PNG, we also investigated the presence of ICAM1-binding motifs and the associated domain architecture of *var* genes among 125 *P. falciparum* isolates from three distinct geographic areas. The results support a role for PfEMP1 variants containing ICAM1-binding DBLβ as targets for protective antimalarial immunity.

RESULTS

Group A PfEMP1 domains are serodominant among young PNG children. Baseline plasma samples from a longitudinal cohort of 187 1- to 3-year-old children from the Maprik area of East Sepik Province, Papua New Guinea, followed for 16 months (25) were screened for antibodies (IgG) to five PfEMP1 domains, including the ICAM1-binding DBL$\beta3_{PF11_0521}$, and four control antigens. They included two domains from a

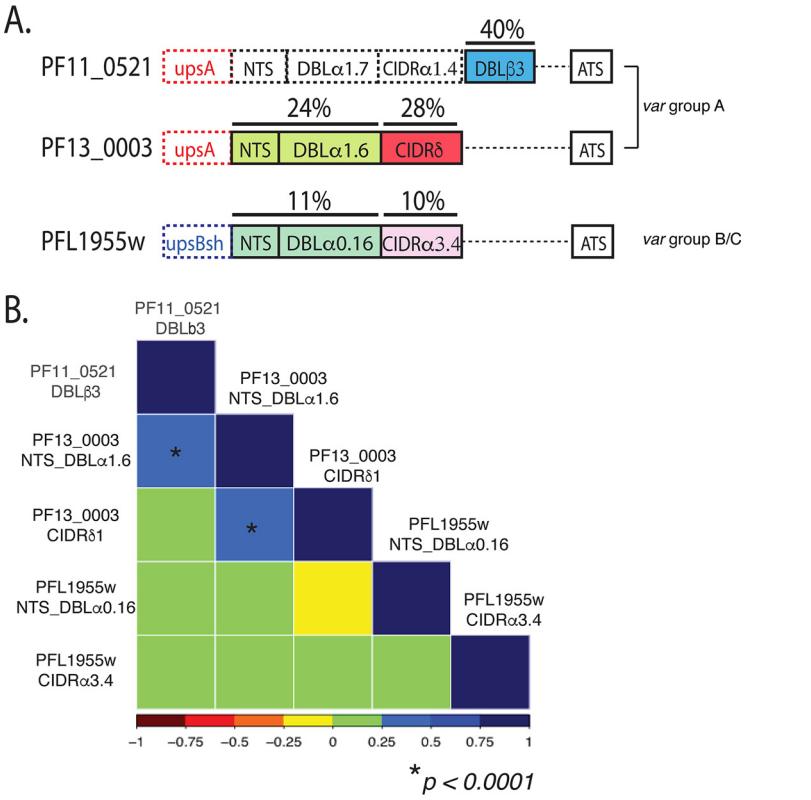


FIG 1 Antibody responses to five PfEMP1 domains in 187 young Papua New Guinean children. (A) Domain compositions and seroprevalences of the five tested PfEMP1 domains. Seroprevalences are indicated as percentages above the relevant domains. ATS indicates the intracellular acidic terminal segment of PfEMP1. (B) Correlation coefficients for seropositivity to five PfEMP1 domains. Significant correlations ($P < 0.001$) are indicated by asterisks.

group A variant, PF13_0003, which has a DBLβ3 domain but does not have an ICAM1-binding motif (NTS-DBLα1.6$_{PF13_0003}$ and CIDRδ_{PF13_0003}) and two domains from a group B/C variant, PFL1955w (NTS-DBLα0.16$_{PFL1955w}$ and CIDRα3.4$_{PFL1955w}$). Among the children, seroprevalence was 2.5- to 4-fold higher for the three type A PfEMP1 domains (40.1% for DBLβ3$_{PF11_0521}$, 27.8% for CIDRδ_{PF13_0003}, and 24.1% for NTS-DBLα1.6$_{PF13_0003}$) than for the type B/C domains (11.2% for NTS-DBLα0.16$_{PFL1955w}$ and 10.1% for CIDRα3.4$_{PFL1955w}$) (Fig. 1A). Pairwise comparisons of antibody responses to the five domains showed that seropositivity to DBLβ3$_{PF11_0521}$ was significantly higher than to all the other domains. In addition, seropositivity to group A domains was significantly higher than to the group B domains (see Table S1 in the supplemental material). Similarly, a combined analysis of seropositivity to any one of the group A PfEMP1s (55%) was significantly higher than the seropositivity to any one of the group B/C domains (18.7%) after correcting for multiple comparisons ($P = 0.003$; Bonferroni adjusted pairwise *t* test).

There was a low but significant correlation between antibody responses to DBLβ3$_{PF11_0521}$ and NTS-DBLα1.6$_{PF13_0003}$ (Spearman's rho [r_s] = 0.36; $P < 0.0001$). This was also the case for the two type A PF13_0003 domains ($r_s = 0.36$; $P < 0.0001$). In contrast, there was no significant correlation between the two group B PFL1955w domains ($r_s = 0.11$; $P = 0.135$) or between domains from the different *var* gene subgroups (Fig. 1B). These low but significant correlations among the group A domains are explained by the predominant expression of and exposure to type A PfEMP1 variants in early childhood infections (23, 24, 28, 29).

Antibodies to five PfEMP1 domains are differentially associated with age and infection status. To investigate whether past and current exposures to malaria influence responses to the five PfEMP1 domains, we investigated associations of antibody

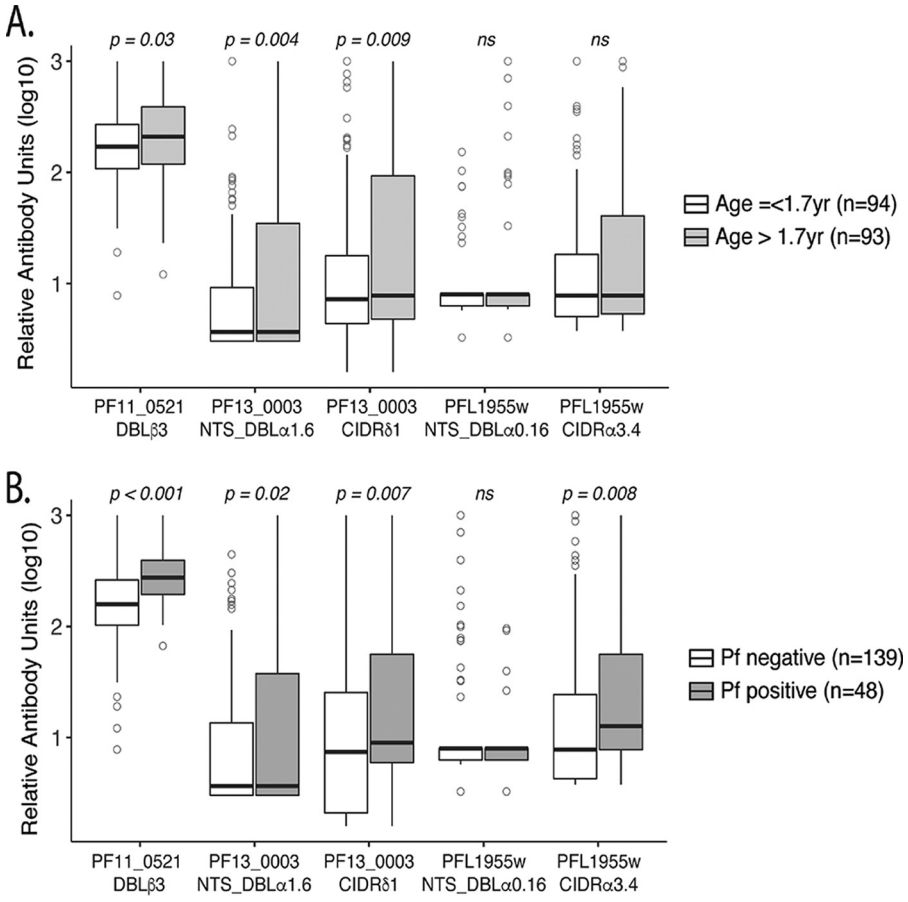


FIG 2 Relationships between antibody responses to five PfEMP1 domains, age, and infection status. (A) IgG levels stratified by age (age groups were determined by the median age [1.7 years]). (B) IgG levels stratified by *P. falciparum* infection status. Box-and-whisker plots are shown for the five PfEMP1 domains. The boxes show the interquartile ranges, the horizontal lines are medians, the whiskers indicate the 95% confidence intervals, and the circles are the outliers (95 to 99%). *P* values for the differences were determined using the Wilcoxon rank sum test between the groups; *ns*, not significant.

levels for each of the domains with age and infection status. Children were split into two groups on the basis of their median age (1.7 years), and median antibody responses were compared. Antibody levels were significantly higher in the older children for group A but not group B/C domains (Fig. 2A). Therefore, the older children had more past exposure to group A antigens than younger children, whereas group B/C domains were similarly recognized irrespective of age. Concurrent microscopic parasitemia (median = 3,349 parasites/μl) at the time of antibody measurement was associated with significantly higher antibody levels than in the noninfected individuals for all domains except group B NTS-DBLα0.16$_{\mathrm{PFL1955w}}$ ($P = 0.94$; Wilcoxon rank sum test) (Fig. 2B), which was poorly reactive overall. Children who were infected at enrollment were 3.43 times more likely to be seropositive to at least one of the five PfEMP1 domains than noninfected children (range, 1.6 to 7.8; $P = 0.002$). Therefore, current infection appeared to boost antibody levels for all antigens across the cohort.

Antibodies to DBLβ3$_{\mathrm{PF11_0521}}$ but not those to other domains are associated with reduced risk of high-density clinical malaria. To examine whether antibody responses against any of the domains were associated with protection against clinical malaria, we conducted a prospective analysis of the risk of febrile episodes and antibody responses. Associations between plasma antibody levels and subsequent risk of symptomatic malaria (all clinical episodes [fever and $\geq$2,500 parasites/μl] and high-density clinical malaria [fever and $\geq$10,000 parasites/μl]) were determined by grouping individuals into terciles and comparing high- and low-antibody groups. An

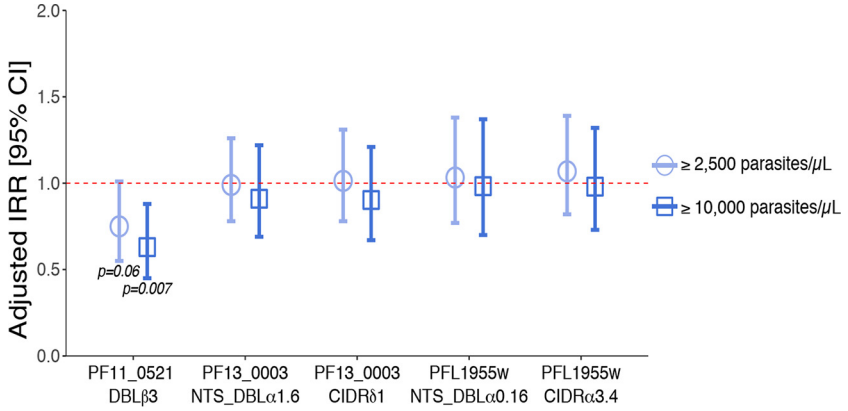


FIG 3 Antibody responses to five PfEMP1 domains and prospective risk of symptomatic malaria. Antibody levels were grouped into three equal groups (high, medium, and low). The incidence rates of clinical malaria and high-density clinical malaria were compared for high and low responders for each tested domain using negative binomial regression. The incidence rate ratios were adjusted for village of residence, seasonal variation, age (continuous), infection status at the time of antibody measurement, and differences in individual exposures ($_{mol}$FOB). The aIRRs for the comparison of high and low responders and the 95% confidence intervals are shown. The *P* values are indicated only when significant ($P < 0.05$).

important feature of the analysis is the adjustment for confounding variables at the individual level, such as the molecular force of blood-stage infection ($_{mol}$FOB) (see Materials and Methods). Children with high levels of antibodies to DBLβ3$_{PF11_0521}$ had a 37% reduction in risk of high-density clinical malaria that was highly significant (febrile illness with ≥10,000 parasites/μl; adjusted incidence rate ratio [aIRR] = 0.63 [95% confidence interval {CI}, 0.45 to 0.88; $P = 0.007$]) and a 25% reduced risk of clinical malaria that was borderline significant (febrile illness with ≥2,500 parasites/μl; aIRR = 0.75 [95% CI, 0.55 to 1.01; $P = 0.06$]). However, there was no significant reduction in risk in either presentation of clinical malaria for the other four domains tested (Fig. 3).

Children who developed severe malaria had significantly lower levels of antibodies to DBLβ3$_{PF11_0521}$. Having determined that antibody responses to DBLβ3$_{PF11_0521}$ were associated with protection against clinical malaria, and in particular high-density clinical malaria, a biomarker for severe disease, we then wanted to examine antibody responses in the children who experienced severe disease in the follow-up period. According to WHO criteria (30), of the 187 children, 18 experienced severe *P. falciparum* malaria during the follow-up period (25) (see Table S2 in the supplemental material). On average, these children were similar to those who did not develop severe malaria with respect to age (severe cases, 1.64 years, versus nonsevere cases, 1.89 years; $P = 0.12$) and exposure, experiencing a similar number of distinct *P. falciparum* infections during the follow-up period (severe, 5.19, and nonsevere, 5.17; $P = 0.98$). However, children who experienced severe malaria had significantly lower levels of antibodies to DBLβ3$_{PF11_0521}$ at baseline than those who did not develop severe malaria (Fig. 4) ($P = 0.004$). Children who developed severe malaria also had significantly lower levels of antibodies to the other type A PfEMP1 domains ($P < 0.01$) and the type B/C domain CIDRα3.4$_{PFL1955w}$ ($P = 0.024$). For the NTS-DBLα0.16$_{PFL1955w}$ domain with low reactivity, there was no significant difference ($P = 0.95$) (Fig. 4).

PNG PfEMP1 proteins with ICAM1-binding DBLβ domains are enriched for DC4 and DC13. The PF11_0521 PfEMP1 variant is from 3D7, an isolate with possible African origin and dual binding activities with ICAM1 (via DBLβ) and EPCR (via DC13) (21). Previous studies investigating dual ICAM1-EPCR binding have focused on collections of reference strains or field isolates from different geographic areas (21, 31). To determine whether PNG isolates possess ICAM-binding motifs and to study the associated PfEMP1 domain architecture, we conducted a detailed analysis of *var* genes extracted from whole-genome sequence data on 125 *P. falciparum* isolates from PNG. Among the

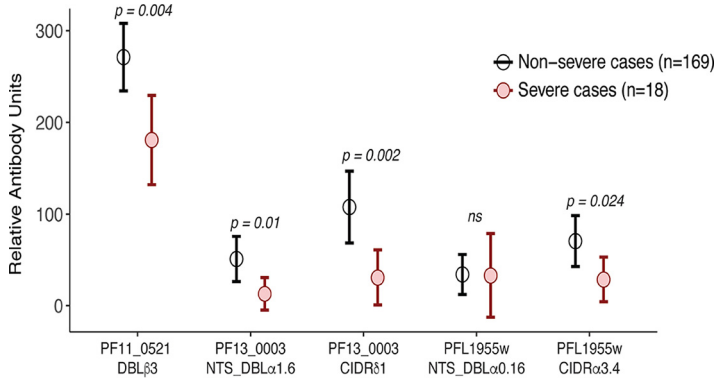
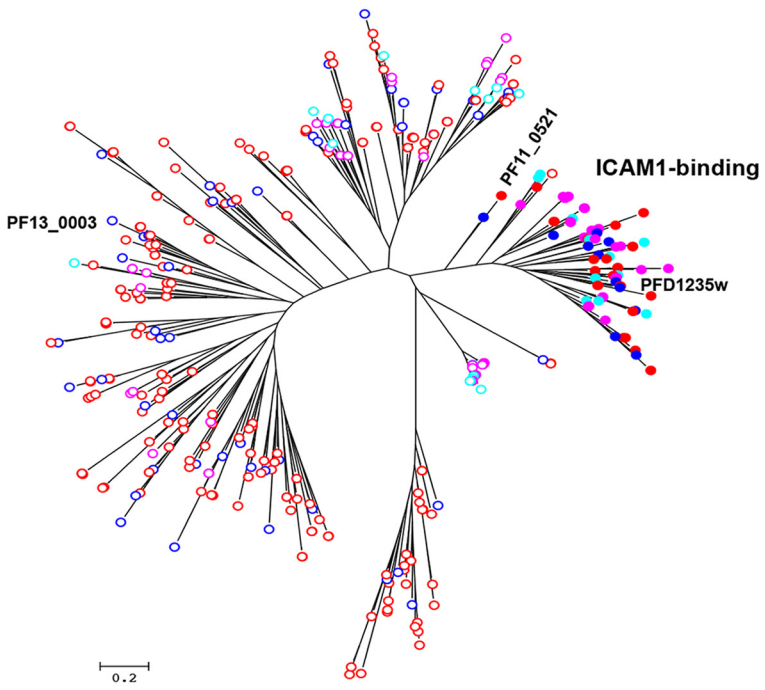


FIG 4 Antibody responses to five PfEMP1 domains and development of severe malaria. Means and standard errors are shown for children who experienced severe malaria and those who did not. *P* values for *t* test comparisons of the means are indicated for each domain.

genomes, we identified 4,044 full or partial open reading frames (ORFs) that were classified as *var* genes using BLAST against a database of classified DBL and CIDR domains (mean number of distinct PfEMP1s per genome = 32 [range: 1 to 60]; mean coverage = 33 reads [range: 8 to 124]) (see Table S3 in the supplemental material). Of those, 117 genomes contained 1,505 DBLβ domains with a mean coverage of 35 reads distributed among 1,420 *var* genes (i.e., many *var* genes had multiple DBLβ domains). For the 8 PNG genomes without DBLβ domains, 6 had low coverage, resulting in poor sampling of *var* genes (n = 1 to 6), while 2 isolates had higher coverage and contained 12 and 21 *var* genes. Among 1,505 PNG DBLβ sequences (see Table S3 and Data Set S1 in the supplemental material), 81 contained the ICAM1-binding motif (21). They included 47 DBLβ1 (58%), 33 DBLβ3 (41%), and 1 DBLβ7 (1%) domains that were distributed among 61 genomes and 80 *var* genes (1 *var* gene had two ICAM1-binding motifs, DBLβ1 and DBLβ7) (see Table S4 in the supplemental material). To examine PNG DBLβ diversity in context with parasite populations of other malaria countries of endemicity, 279 DBLβ sequences from other countries and reference isolates, including DBL$\beta3_{PF11_0521}$ (linked to DC13) and DBL$\beta3_{PFD1235w}$ (another ICAM1-binding variant linked to DC4 [18, 19]), were included in the analysis (see Data Set S1 in the supplemental material). Of these, 22 contained the ICAM1-binding motif, and they were found within 11 DBLβ1 (50%) and 11 DBLβ3 (50%) domains (see Table S5 in the supplemental material). All DBLβ1 and -β3 domains from both data sets (406 PNG plus 178 other) (see Data Sets S1 and S2 in the supplemental material) were then combined for multiple alignments. Truncated sequences were removed, resulting in 455 sequences, including 102 with the ICAM1 motif. Phylogenetic analysis of the subdomain 3 (SD3) region encompassing the ICAM1-binding motif revealed an "ICAM1-binding" cluster populated entirely by DBLβ with the ICAM1-binding motif (Fig. 5A). The average evolutionary distance of the DBLβ predicted to bind ICAM1 was 0.532 compared to 1.011 for the remaining DBLβs. PNG DBLβ sequences were distributed throughout the tree, and there was no evidence of population structure. Of note, PF13_0003 contains a DBLβ3 sequence; however, it did not contain an ICAM1-binding motif and was divergent from sequences in the ICAM1-binding clade (Fig. 5A). These results suggest that the majority of PNG parasites carry at least one PfEMP1 with predicted ICAM1 binding.

To determine the PfEMP1 context of DBLβ with predicted ICAM1 binding, we investigated the domain architecture of full-length *var* gene assemblies from the PNG genomic sequence data with respect to adjacent domains and specific DC classes. All PNG PfEMP1 sequences with DBLβ1 and -β3 domains contained CIDRα1 domains (EPCR binding). However, PfEMP1 proteins containing DBLβ domains with the ICAM1 motif (n = 80) (see Tables S3 and S4 in the supplemental material) were significantly enriched for adjacent DC13 structures (46.3% compared to 20.5% among PfEMP1 proteins with DBLβ domains with no motif; $P < 0.0001$; binomial exact test) and DC4 (13.8%

A.



B.

							ICAM1 motif	*All*	*p-value*
DC4	UpsA	NTS	DBLα1.1/1.4	CIDRα1.6	DBLβ1/3	ATS	11 (13.8%)	20 (6.0%)	0.008
DC8	UpsA	NTS	DBLα2	CIDRα1.1/1.8	DBLβ1/3	ATS	0 (0%)	27 (8.2%)	0.002
DC13	UpsA	NTS	DBLα1.7	CIDRα1.4	DBLβ1/3	ATS	37 (46.3%)	68 (20.5%)	<0.0001
DC16	UpsA	NTS	DBLα1.5/1.6	CIDR$\beta/\delta/\gamma$	DBLβ3	ATS	0 (0%)	30 (9.1%)	<0.0001
DC1-VAR1	UpsA	NTS	DBLα1.4	CIDRα1.3	DBLβ1	ATS	0 (0%)	38 (11.5%)	0.0001
					Others		22	148	n.a.
	known ICAM1 binding DBLβ				**Unknown**		10	0	n.a.
					TOTAL		**80**	**331**	**n.a.**

FIG 5 Conservation of ICAM1-binding motifs and dual EPCR-ICAM1-binding cassettes in Papua New Guinea. *var* gene sequences were assembled for 125 *P. falciparum* isolates from Papua New Guinea, and domains were classified as previously described (42). (A) Maximum likelihood tree of 473 DBLβ1 and DBLβ3 sequences, including 406 from PNG (pink, DBLβ1; red, DBLβ3) and 67 from isolates from diverse geographic locations (cyan, DBLβ1; blue, DBLβ3). Sequences containing the minimal ICAM1-binding motif are indicated by solid circles. Genes described in this study are labeled at the DBLβ1/3 variant positions in the tree. (B) Domain architecture of PNG *var* genes containing DBLβ1/3 domains with ICAM1 motifs. The presence of domain cassettes among the 80 *var* genes containing DBLβ1/3 with the ICAM1-binding motif relative to that among a subset of 331 PNG *var* genes containing any DBLβ1/3 is indicated on the right; n.a., not applicable. Significance was determined by a binomial exact test.

compared to 6.0%; $P = 0.008$; binomial exact test) (Fig. 5B; see Table S6 in the supplemental material), which are strongly associated with severe malaria and dual EPCR/ICAM1 binding (21, 31). In the phylogenetic analysis, DBLβs with adjacent DC4 and DC13 were divergent, although several DBLβs associated with DC4 were identical, suggesting conservation of a common *var* gene (see Fig. S2 in the supplemental material). Other DC structures, including DC8, DC16, and DC1-var1, were not found among the PfEMP1 proteins with the ICAM1-binding motif (Fig. 5B). This suggests that specific classes of PfEMP1 proteins with dual binding specificities for ICAM1 and EPCR are maintained in PNG isolates, confirming previous observations in African isolates (21).

DISCUSSION

PF11_0521 belongs to a class of group A PFEMP1 proteins with DBLβ domains shown to bind ICAM1 and found exclusively in PfEMP1 proteins with EPCR-binding CIDRα1 (16, 32). These domains are adjacent to each other, and together, they may produce a binding phenotype often associated with sequestration leading to cerebral

malaria (13, 18–21, 31). Our results support a role for these PfEMP1 proteins in clinical and severe malaria in young children by demonstrating that (i) antibodies against DBLβ3$_{PF11_0521}$ are significantly associated with protection against high-density clinical malaria, (ii) children who developed severe malaria had lower levels of antibodies to DBLβ3$_{PF11_0521}$ prior to the disease episode (albeit antibodies to other antigens were also lacking in these children), (iii) there are ICAM1-binding motifs present in DBLβ sequences from PNG isolates, and (iv) PfEMP1/*var* genes with predicted ICAM1 binding are also predicted to bind EPCR (21, 31).

The cohort of 1- to 3-year-old PNG children was specifically chosen to explore early antibody responses to PfEMP1, because their immunity to malaria was incomplete (25), and to limit the complex background antibody responses that are observed in older children and adults (27). Antibodies to PfEMP1 domains were associated with age and current infection in the cohort, consistent with exposure driving the acquisition and maintenance of immunity to malaria (2). In addition to the measurement of exposure to new infections in the follow-up period ($_{mol}$FOB), these results provided a basis for exploring associations with the risk of disease adjusted for important confounding factors (33). Because the number of severe disease cases was small ($n = 18$), we initially focused the prospective risk analysis on clinical infections, which occurred at a high rate in these children (25). High-density clinical malaria (fever plus $\geq$10,000 parasites/μl) is considered a surrogate marker for severe disease, since the children have intense infections, yet are not classified into any of the severe-disease syndromes by WHO criteria (30). The significant reduction in risk of these high-density clinical infections if the children had high levels of DBLβ3$_{PF11_0521}$ antibodies suggests that inhibition of ICAM1 binding or other binding phenotypes by antibodies against these PfEMP1 proteins may limit the parasite burden and progression to severe malaria. Antibodies to DBLβ3$_{PF11_0521}$ have previously been associated with a reduced risk of hospitalization with severe or moderately severe malaria in Tanzanian children (22), clearly demonstrating a potentially protective immune response associated with exposure to PfEMP1 with DBLβ3$_{PF11_0521}$-like variants. Tanzanian children with high antibody reactivity to CIDRα1 from the other confirmed ICAM1-binding protein, PFD1235w, also had a lower risk of anemia (hemoglobin, $<$11 g/dl) and clinical malaria (34). Furthermore, ICAM1-binding-inhibitory antibodies are common in hyperimmune adults living in areas of endemicity (19), suggesting that they may play an important role in the maintenance of clinical immunity. The mechanism of protection may be through direct inhibition of ICAM1 binding, as shown in *in vitro* experiments (21, 32); indirectly, through prevention of binding of other domains, such as EPCR-binding CIDRα1 (12); or synergistic antibody responses targeting multiple domains.

While a broad repertoire of PfEMP1 antibodies was acquired in these young children, only antibodies against the ICAM1-binding full-length DBLβ3$_{PF11_0521}$ domain were associated with protection against both clinical and severe malaria. The results are strengthened by the fact that children also acquired antibodies to domains from the other group A PfEMP1 variants tested in this study (NTS-DBLα1.6$_{PF13_0003}$ and CIDRδ_{PF13_0003}), yet they were not associated with a reduced risk of clinical malaria. The higher recognition of group A antigens in the children overall and the significantly higher recognition in older children for group A, but not group B/C, antigens suggest shared epitopes within this group of PfEMP1 variants and confirm a hierarchy of PfEMP1 exposure with age (27–29, 35). One caveat is the use of different assays for DBLβ3$_{PF11_0521}$ and the control antigens, which prevented the direct comparison of antibody units between antigens. However, the prospective risk analyses and comparison between groups were done for each antigen independently, thus limiting potential biases of the different assays. Another limitation of this study is the small number of PfEMP1 proteins and the lack of a direct comparison of DBLβ3$_{PF11_0521}$ with other non-ICAM1-binding DBLβ domains. Parallel analyses of antibody responses to a large panel and variety of PfEMP1 domains will be a valuable extension of this study.

Dissecting the association of antibodies to different parasite antigens with protection and exposure is important in understanding naturally acquired immunity to

malaria (33). In the same cohort, high levels of antibodies to merozoite antigens were predictive of an increased risk of developing clinical malaria (33). In older PNG children aged 5 to 14 years, however, antibodies against merozoite antigens were found to be associated with protection against clinical malaria (33). Therefore, merozoite antigens were biomarkers of accumulated malaria exposure in the younger age group; however, with increasing exposure and responses of higher magnitude, antibodies to merozoite antigens became biomarkers of protective immunity (33). We used $_{mol}$FOB as a marker of exposure at an individual level to adjust for the confounding effects of exposure in the prospective risk analysis (26, 33). We observed significant protection against clinical malaria in children with high levels of DBLβ3$_{PF11_0521}$ antibodies despite these differing exposures to malaria. That is, children who had antibodies to DBLβ3$_{PF11_0521}$ had lower rates of clinical malaria than those who had low levels of these antibodies, even after adjusting for individual differences in the rates of new malaria infections.

We also found an association between the lack of antibodies to DBLβ3$_{PF11_0521}$ and other group A PfEMP1 domains and the prospective risk of severe malaria; however, in this analysis, we also found associations with other antigens. Children who experienced only uncomplicated or asymptomatic malaria had significantly higher levels of antibodies to all three group A antigens and one of the group B antigens than children who developed severe malaria in the follow-up period. Altogether, the observed association of antibodies to DBLβ3$_{PF11_0521}$ with reduced risk of high-density clinical malaria and severe malaria suggests epitopes in DBLβ3$_{PF11_0521}$-like sequences or adjacent PfEMP1 domains may be important targets of protective immunity. The association with other domains in severe malaria may be due to the fact that we did not adjust for confounders and is also consistent with the early acquisition (and potential protective effects) of antibody responses to group A antigens compared to B antigens (27, 28, 35). However, we cannot rule out the possibility that other domains tested are important targets of protection against severe malaria.

The importance of host-parasite interactions via ICAM1 to the PNG parasite population is indicated by the maintenance of a class of relatively conserved DBLβ sequences with predicted ICAM1 binding (18, 21). Phylogenetic analysis of the C-terminal DBL SD3 of DBLβ sequences extracted from 125 parasite genomes from PNG, together with those of geographically diverse isolates, identified a cluster of sequences previously shown (19, 21) or predicted to bind ICAM1 as distinguished by a common sequence motif (21). Our data suggest that similar subsets of group A PfEMP1 proteins with ICAM1-binding DBLβs are found in PNG parasites. The lower diversity of this group of sequences compared to other non-ICAM1-binding DBLβ1/3 suggests positive selection due to functional specialization for binding to ICAM1. We also found that DBLβ domains with ICAM1-binding motifs are located adjacent to domain cassettes associated with EPCR binding and severe disease (DC13 and DC4) (18, 20, 21). This assemblage is predicted to confer a dual-binding phenotype that has been associated with severe malaria (12, 21). These results are consistent with the conservation of these specialized classes of PfEMP1 proteins across large geographic distances, since previous studies have focused on African parasites or reference isolates from diverse locations (18, 21, 31). The high reactivity of children's sera to the full-length DBLβ_{PF11_0521} supports the notion that conserved epitopes exist that can be targeted by cross-reactive and protective antibodies (18, 21, 32). However, the ICAM1-binding motif is located in the C-terminal part of the protein (SD3 region), and no sequence traits in the N-terminal part of the domain have been linked to ICAM1 binding. The protective association may therefore be accounted for by epitopes outside the SD3 region or as a result of its concurrence with other virulence-associated domains in the same PfEMP1.

Our analysis of antibodies to functionally diverse PfEMP1 domains extends previous insights into early exposure to PfEMP1 (22, 27, 28, 35), revealing that young children from PNG are highly exposed to group A antigens while having limited exposure to group B/C antigens. The finding that high levels of antibodies against DBLβ3$_{PF11_0521}$ are associated with a reduced risk of high-density clinical and severe malaria supports a role for PfEMP1 in malaria pathogenesis via ICAM1-binding domains or adhesion of

adjacent domains. The demonstration that PNG *P. falciparum* isolates contain PfEMP1/*var* genes with predicted ICAM1 binding closely linked to predicted EPCR-binding CIDR domains suggests positive selection and functional specialization of a subclass of dual-binding PfEMP1 proteins implicated in severe malaria syndromes. Studies investigating whether antibodies in clinically immune children interrupt binding interactions between ICAM1 and this class of DBLβ sequences would establish a more direct link to protection against malaria. Antibodies to the EPCR-binding CIDRα1 domains cooccurring with ICAM1-binding DBLβ may have synergistic protective effects; however, this is yet to be established. This study adds to the growing body of evidence supporting the development of specific classes of PfEMP1 proteins as vaccine candidates. Furthermore, it suggests that this class of DBLβ domains could be used as diagnostic antigens to track population immunity during malaria elimination.

MATERIALS AND METHODS

Cohort study design. Plasma samples were collected during a longitudinal cohort survey conducted in the East Sepik Province of PNG. A detailed description of the study has been published elsewhere (25, 26). Briefly, 190 children aged 1 to 3 years were enrolled at the start of the study in March 2006, and 74 additional children were enrolled over the following 6 months. The children were followed for 69 weeks with active and passive follow-up (25). The children were visited fortnightly, with collection of 2 blood samples 24 h apart for active detection of malaria infection every 8 to 9 weeks. The demographic and clinical characteristics of the study population and incidences of clinical malaria and severe malaria in each 8- to 9-week follow-up interval have been described in detail elsewhere (25, 26). Antibody assays were performed on plasma samples collected from 187 of the 190 children enrolled in March 2006. Of the 187 children, 48 (25.6%) were microscopy positive for *P. falciparum*. The average $_{mol}$FOB in this subset of the cohort was 5.2, and the average number of clinical episodes was 2 per child per year at risk (25, 26). *P. falciparum* clinical episodes were defined as febrile illness (axillary temperature of $\geq$37.5°C or history of fever in the preceding 48 h) and $>$2,500 parasites/μl. A high-density clinical episode was defined as febrile illness and $>$10,000 parasites/μl. The characteristics of children with severe malaria are summarized in Table S2 in the supplemental material. Written informed consent was obtained before enrollment of each child. Ethical approval for the study was granted by the PNG Institute of Medical Research (10.21), the Medical Research Advisory Council of PNG (10.55), and the Walter and Eliza Hall Institute of Medical Research (11.03).

Protein expression, purification, and refolding. DBLβ3$_{PF11_0521}$ (also known as DBL2β_{PF11_0521}) was expressed, purified, and refolded as described previously (17). As control PfEMP1 proteins without ICAM1-binding activity, we selected NTS-DBLα and CIDR domains of two *var* genes of 3D7. PF13_0003 is a group A PfEMP1 that has been associated with the formation of rosettes, a phenotype linked with severe malaria (36). In contrast, PFL1955w is a group B/C PfEMP1 with limited antibodies acquired in young children (36). The sequences of all five domains are available in Text S1 in the supplemental material.

NTS-DBLα1.6$_{PF13_0003}$, CIDRδ_{PF13_0003}, NTS-DBLα0.16$_{PFL1955w}$ and CIDRα3.4$_{PFL1955w}$ codon-optimized sequences were synthesized for *Escherichia coli* expression (GeneArt). The GenBank accession numbers are PF13_0003 (XM_001349704) and PFL1955w (XM_001350761). Sequences were excised from the supplier's vector using BamHI and XhoI and ligated into the pProExHTb expression vector (Invitrogen), which incorporates an N-terminal hexahistidine fusion tag. The vectors were then transformed into *E. coli* strain BL21(DE3) for expression, as described previously (37). Briefly, the transformed cultures were grown in superbroth, expression was induced with 1 mm isopropyl 1-thio-β-D-galactopyranoside, and the cultures were grown for a further 3 h at 37°C. The cells were harvested by centrifugation, lysed by sonication, and processed as either insoluble inclusion bodies (NTS-DBLα1.6$_{PF13_0003}$, CIDRδ_{PF13_0003}, and NTS-DBLα0.16$_{PFL1955w}$) or soluble proteins (CIDRα3.4$_{PFL1955w}$).

NTS-DBLα1.6$_{PF13_0003}$, CIDRδ_{PF13_0003}, and NTS-DBLα0.16$_{PFL1955w}$ proteins were deposited as insoluble inclusion bodies. Cells were lysed by sonication, and the inclusion bodies were solubilized by the addition of 6 M guanidine HCl, pH 8.0. The solubilized proteins were purified by metal-chelating chromatography using a nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen) under reducing conditions. Optimum refolding conditions were determined for each protein. The Ni-NTA-eluted CIDRα3.4$_{PFL1955w}$ and the refolded CIDRδ_{PF13_0003} proteins were purified using strong anion-exchange chromatography. The proteins were eluted from a HiTrap Q column (GE Healthcare), and the relevant fractions were pooled and concentrated. For CIDRα3.4$_{PFL1955w}$, the protein was further purified by size exclusion chromatography. The refolded NTS-DBLα1.6$_{PF13_0003}$ and NTS-DBLα0.16$_{PFL1955w}$ domains were further purified using cation-exchange chromatography. The bound proteins were eluted from a HiTrap SP column (GE Healthcare), and the relevant fractions were pooled, concentrated, and further purified by size exclusion chromatography. The purity of each protein was assessed on SDS-PAGE gels and via Western immunoblots under standard conditions. Briefly, proteins were run on a 4 to 12% Bis-Tris SDS-PAGE (Invitrogen). Standard Western blotting procedures were performed for nonreduced and reduced (by addition of β-mercaptoethanol) samples using nitrocellulose, and the immunoblots were processed with enhanced chemiluminescence (ECL) substrates (GE Healthcare). For all Western blots, recombinant proteins were detected with pooled hyperimmune sera from highly exposed PNG adults (see Fig. S1 in the supplemental material). A single batch of each protein was used for all serological screening. The purified

proteins were assessed using a pool of hyperimmune plasma (see Fig. S1 in the supplemental material), and a single batch of each protein was used for all serological screening.

Measurement of antibody responses. Plasma samples collected at enrollment ($n = 187$) were tested for antibodies comprising total immunoglobulin G (IgG) to DBL$\beta 3_{PF11_0521}$ using a standard enzyme-linked immunosorbent assay (ELISA). For the other four domains, IgG levels were measured using a cytometric bead array (CBA) as described previously (38). The details are described below.

ELISAs were performed to measure total IgG using standard methods. Ninety-six-well plates (Nunc, Denmark) were coated with 1 μg/ml of DBL$\beta 3_{PF11_0521}$ recombinant protein in phosphate-buffered saline (PBS) and incubated overnight at 4°C. PBS with 5% skim milk was used for blocking and PBS with 1% skim milk and 0.05% Tween for diluting the plasma samples and antibodies. Plasma was added at 1-in-100 dilutions. For measurement of total IgG, horseradish peroxidase-conjugated mouse anti-human IgG (Southern Biotech, USA) was used at a dilution of 1 in 1,000. Finally, TMB microwell peroxidase substrate (KPL, Inc., Australia) was added, the reaction was stopped using 1 M H_3PO_4, and the optical density (OD) was measured at 450 nm. All the samples were tested in duplicate. Background (determined from wells with no plasma) was deducted, and the threshold for a seropositive response was determined using reactivities of 1:100-diluted plasma samples from anonymous malaria-naive Australian adults ($n = 12$). The mean value among these negative-control plasma samples plus 3 standard deviations (SD) was used as a cutoff value to define seropositivity.

CBAs were carried out using four sets of microbeads (BD Bioscience, San Diego, CA, USA) with distinct and nonoverlapping fluorescence intensities covalently coupled to NTS-DBL$\alpha 1_{PF13_0003}$, CIDRδ_{PF13_0003}, NTS-DBL$\alpha 0.16_{PFL1955w}$, and CIDR$\alpha 3.4_{PFL1955w}$ recombinant proteins according to the manufacturer's protocol. Briefly, 150 μl of selected microbeads was sonicated for 1 min and incubated with 3.8 μl of 1 M dithiothreitol (DTT) for 1 h at room temperature with agitation. The beads were washed 3 times and resuspended in 40 μl of coupling buffer (BD Bioscience). Recombinant proteins (1-mg/ml concentration) were activated by incubation with 4 μl of sulfosuccinimidyl 4-*N*-maleimidomethyl cyclohexane 1-carboxylate (2 mg/ml) for 1 h. The protein mixture was then run through a buffer exchange spin column (Bio-Rad) preequilibrated with the coupling buffer (BD Bioscience). The activated protein was added to the washed microbeads and allowed to conjugate for 1 h at room temperature with agitation. Four microliters of *N*-ethylmaleimide (2 mg/ml) was added, and the mixture was incubated for another 15 min. The conjugated microbeads were then washed, resuspended in 1 ml of storage buffer (BD Bioscience), and kept at 4°C in the dark. For assessment of antibody responses, 1 μl of conjugated microbeads was diluted in 50 μl of washing buffer (BD Bioscience) containing 1-in-100 dilutions of plasma samples. Duplicate samples were then incubated for 1 h at room temperature in the dark, washed, and further incubated with mouse anti-human IgG phycoerythrin (PE)-conjugated antibody (BD Bioscience) for 1 h at room temperature. After washing, the samples were resuspended in diluent buffer containing PE (BD Bioscience) and acquired using an LSR Fortessa analyzer (Becton Dickinson, New Jersey, USA). Analysis was performed using FlowJo software, and the median fluorescence intensity (MFI) for each bead (recombinant protein) was calculated. Background (determined from the unconjugated beads with plasma samples and conjugated beads with no plasma samples) was deducted from the mean of each sample. The threshold for a seropositive response was determined using reactivities of 1:100-diluted plasma samples from anonymous malaria-naive Australian adults ($n = 12$). The mean value among these negative-control plasma samples plus 3 SD was considered seropositive. The analysis was done independently for each antigen.

A serial dilution of plasma samples from a pool of hyperimmune PNG adults was included in each plate to determine standard curves, which were later fitted using a 5-parameter logistic regression model (39) to transform antibody measured by the two assays into relative antibody units and to correct plate-to-plate variations within an assay.

Statistical analysis. Statistical analyses were performed using STATA version 12.1 software (Stata Corporation, USA). Differences in median antibody levels by age group and *P. falciparum* infection status were compared using the Wilcoxon rank sum test. The proportions of children seropositive to different domains were compared using chi-square tests. Correlation coefficients for antibody levels were determined using Pearson's correlation.

Analyses of the cohort data showed significant overdispersion in the number of clinical episodes per child (25), and as a result, a negative binomial model with generalized estimating equations (GEE) (based on the xtnberg function in STATA) with an exchangeable correlation structure and a semirobust variance estimator was used for the analyses of the association of antibody levels and the incidence of clinical (fever with $\geq$2,500 parasites/μl) and high-density clinical (febrile illness and $\geq$10,000 parasites/μl) episodes during the follow-up period. Antibody levels were grouped into terciles (low, medium, and high responses), and their association with clinical and high-density clinical episodes was assessed by univariate analyses adjusted for seasonal variation, village of residence, age at the time of enrollment, *P. falciparum* infection status, and individual exposure, as measured by the $_{mol}$FOB. The $_{mol}$FOB is the number of genetically distinct *P. falciparum* clones (based on *msp2* genotyping) each child acquired per year at risk (26).

Sequence analyses. To identify ICAM1-binding DBLβ domains and corresponding full-length PfEMP1 sequences in the PNG parasite population, we extracted *var* gene sequences from the genomes of 125 clinical *P. falciparum* isolates collected from three distinct geographic areas of PNG. These isolates were sequenced as part of the MalariaGEN Community Project (European Nucleotide Archive [ENA] accession numbers are listed in Table S3 in the supplemental material). Illumina short-read data sequences were assembled using Velvet version 1.2.03 (40). Open reading frames were extracted using Virtual Ribosome ORF finder (41), and *var* gene-encoded PfEMP1 domain sequences were extracted from

these by BLAST using a library of previously annotated PfEMP1 domain sequences from 7 reference genomes and classified into subgroups as previously described (42). All DBLβ domains were extracted from the PNG data set (n = 1,505 [see Data Set S1 in the supplemental material]) and complemented with a supplementary data set from 226 assembled genomes sequenced in the MalariaGEN Community Project (43) and 7 reference genomes (42), including confirmed ICAM1-binding DBLβ domains of PF11_0521 and PFD1235w (18) (n = 279) (see Data Set S2 in the supplemental material). DBLβ sequences were screened for a relaxed version of the ICAM1 motif, N-G-G-[PA]-x-Y-x(27)-G-P-P-x(3)-H (21), using the Web-based server Scan Prosite (44). All sequences from the DBLβ classes with ICAM1-binding motifs (DBLβ1 and DBLβ3) were aligned using Muscle with default settings in MEGA version 7.0 (37). After removing truncated sequences (n = 40) and focusing on the S3 region of DBLβ, which contains the ICAM1-binding motif (21), we then conducted a phylogenetic analysis using a total of 367 PNG DBLβ1 and DBLβ3 sequences originating from PNG isolates, together with 89 DBLβ1 and DBLβ3 sequences from the supplementary data set. A maximum likelihood tree was estimated using the JTT substitution model with 1,000 bootstrap repetitions in MEGA version 7.0 (45). We measured the average evolutionary divergence among sequences using the Dayhoff model in MEGA version 7.0 (45). In addition, we characterized the domain architecture of PfEMP1 with DBLβ1 and DBLβ3 domains by extracting the domain classifications from the BLAST output (see Table S6 in the supplemental material). We then assessed whether the frequency of each domain cassette among the ICAM1-motif containing sequences varied significantly from the expected frequency (among a subset of 331 PfEMP1 proteins with DBLβ1/3 domains) using an exact binomial test with R software (46).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00485-17.

SUPPLEMENTAL FILE 1, PDF file, 2.1 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 3, CSV file, 0.7 MB.
SUPPLEMENTAL FILE 4, CSV file, 0.1 MB.

ACKNOWLEDGMENTS

We thank all participants in the study and their parents or guardians, and staff involved in the study at the Papua New Guinea Institute of Medical Research.

This research was supported by the National Health and Medical Research Council of Australia (NHMRC grant numbers GNT1005653 and GNT1027109 to A.E.B.) and the Danish Council for Independent Research (DFF-4004-00624B). Production of DBL$\beta3_{PF11_0521}$ protein was supported by a U.S. National Institutes of Health grant R01AI092120 to A.V.O. I.M. is supported by an NHMRC Senior Research Fellowship. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

REFERENCES

1. WHO. 2016. World malaria report. WHO, Geneva, Switzerland.
2. Bull PC, Lowe BS, Kortok M, Molyneux CS, Newbold CI, Marsh K. 1998. Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. Nat Med 4:358–360. https://doi.org/10.1038/nm0398-358.
3. Voss TS, Healer J, Marty AJ, Duffy MF, Thompson JK, Beeson JG, Reeder JC, Crabb BS, Cowman AF. 2006. A *var* gene promoter controls allelic exclusion of virulence genes in Plasmodium falciparum malaria. Nature 439:1004–1008.
4. Chan JA, Howell KB, Reiling L, Ataide R, Mackintosh CL, Fowkes FJ, Petter M, Chesson JM, Langer C, Warimwe GM, Duffy MF, Rogerson SJ, Bull PC, Cowman AF, Marsh K, Beeson JG. 2012. Targets of antibodies against Plasmodium falciparum-infected erythrocytes in malaria immunity. J Clin Invest 122:3227–3238. https://doi.org/10.1172/JCI62182.
5. Su XZ, Heatwole VM, Wertheimer SP, Guinet F, Herrfeldt JA, Peterson DS, Ravetch JA, Wellems TE. 1995. The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes. Cell 82:89–100. https://doi.org/10.1016/0092-8674(95)90055-1.
6. Smith JD, Chitnis CE, Craig AG, Roberts DJ, Hudson-Taylor DE, Peterson DS, Pinches R, Newbold CI, Miller LH. 1995. Switches in expression of Plasmodium falciparum var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. Cell 82:101–110. https://doi.org/10.1016/0092-8674(95)90056-X.
7. Baruch DI, Pasloske BL, Singh HB, Bi X, Ma XC, Feldman M, Taraschi TF, Howard RJ. 1995. Cloning the P. falciparum gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. Cell 82:77–87. https://doi.org/10.1016/0092-8674(95)90054-3.
8. Scherf A, Hernandez-Rivas R, Buffet P, Bottius E, Benatar C, Pouvelle B, Gysin J, Lanzer M. 1998. Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intraerythrocytic development in Plasmodium falciparum. EMBO J 17:5418–5426. https://doi.org/10.1093/emboj/17.18.5418.
9. Scherf A, Lopez-Rubio JJ, Riviere L. 2008. Antigenic variation in Plasmodium falciparum. Annu Rev Microbiol 62:445–470. https://doi.org/10.1146/annurev.micro.61.080706.093134.
10. Srivastava A, Gangnard S, Round A, Dechavanne S, Juillerat A, Raynal B, Faure G, Baron B, Ramboarina S, Singh SK, Belrhali H, England P, Lewit-Bentley A, Scherf A, Bentley GA, Gamain B. 2010. Full-length extracellular region of the var2CSA variant of PfEMP1 is required for specific, high-affinity binding to CSA. Proc Natl Acad Sci U S A 107:4884–4889. https://doi.org/10.1073/pnas.1000951107.
11. Baruch DI, Ma XC, Singh HB, Bi X, Pasloske BL, Howard RJ. 1997. Identification of a region of PfEMP1 that mediates adherence of Plasmodium

falciparum infected erythrocytes to CD36: conserved function with variant sequence. Blood 90:3766–3775.
12. Turner L, Lavstsen T, Berger SS, Wang CW, Petersen JE, Avril M, Brazier AJ, Freeth J, Jespersen JS, Nielsen MA, Magistrado P, Lusingu J, Smith JD, Higgins MK, Theander TG. 2013. Severe malaria is associated with parasite binding to endothelial protein C receptor. Nature 498:502–505. https://doi.org/10.1038/nature12216.
13. Smith JD, Craig AG, Kriek N, Hudson-Taylor D, Kyes S, Fagen T, Pinches R, Baruch DI, Newbold CI, Miller LH. 2000. Identification of a Plasmodium falciparum intercellular adhesion molecule-1 binding domain: a parasite adhesion trait implicated in cerebral malaria. Proc Natl Acad Sci U S A 97:1766–1771. https://doi.org/10.1073/pnas.040545897.
14. Lavstsen T, Salanti A, Jensen AT, Arnot DE, Theander TG. 2003. Subgrouping of Plasmodium falciparum 3D7 var genes based on sequence analysis of coding and non-coding regions. Malar J 2:27. https://doi.org/10.1186/1475-2875-2-27.
15. Smith JD, Rowe JA, Higgins MK, Lavstsen T. 2013. Malaria's deadly grip: cytoadhesion of Plasmodium falciparum-infected erythrocytes. Cell Microbiol 15:1976–1983. https://doi.org/10.1111/cmi.12183.
16. Bull PC, Abdi AI. 2016. The role of PfEMP1 as targets of naturally acquired immunity to childhood malaria: prospects for a vaccine. Parasitology 143:171–186. https://doi.org/10.1017/S0031182015001274.
17. Gullingsrud J, Saveria T, Amos E, Duffy PE, Oleinikov AV. 2013. Structure-function-immunogenicity studies of PfEMP1 domain DBL2betaPF11_0521, a malaria parasite ligand for ICAM-1. PLoS One 8:e61323. https://doi.org/10.1371/journal.pone.0061323.
18. Bengtsson A, Joergensen L, Rask TS, Olsen RW, Andersen MA, Turner L, Theander TG, Hviid L, Higgins MK, Craig A, Brown A, Jensen AT. 2013. A novel domain cassette identifies Plasmodium falciparum PfEMP1 proteins binding ICAM-1 and is a target of cross-reactive, adhesion-inhibitory antibodies. J Immunol 190:240–249. https://doi.org/10.4049/jimmunol.1202578.
19. Oleinikov AV, Amos E, Frye IT, Rossnagle E, Mutabingwa TK, Fried M, Duffy PE. 2009. High throughput functional assays of the variant antigen PfEMP1 reveal a single domain in the 3D7 Plasmodium falciparum genome that binds ICAM1 with high affinity and is targeted by naturally acquired neutralizing antibodies. PLoS Pathog 5:e1000386. https://doi.org/10.1371/journal.ppat.1000386.
20. Turner GD, Morrison H, Jones M, Davis TM, Looareesuwan S, Buley ID, Gatter KC, Newbold CI, Pukritayakamee S, Nagachinta B, White NJ, Berendt AR. 1994. An immunohistochemical study of the pathology of fatal malaria. Evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration. Am J Pathol 145:1057–1069.
21. Lennartz F, Adams Y, Bengtsson A, Olsen RW, Turner L, Ndam NT, Ecklu-Mensah G, Moussiliou A, Ofori MF, Gamain B, Lusingu JP, Petersen JE, Wang CW, Nunes-Silva S, Jespersen JS, Lau CK, Theander TG, Lavstsen T, Hviid L, Higgins MK, Jensen AT. 2017. Structure-guided identification of a family of dual receptor-binding PfEMP1 that is associated with cerebral malaria. Cell Host Microbe 21:403–414. https://doi.org/10.1016/j.chom.2017.02.009.
22. Oleinikov AV, Voronkova VV, Frye IT, Amos E, Morrison R, Fried M, Duffy PE. 2012. A plasma survey using 38 PfEMP1 domains reveals frequent recognition of the Plasmodium falciparum antigen VAR2CSA among young Tanzanian children. PLoS One 7:e31011. https://doi.org/10.1371/journal.pone.0031011.
23. Jespersen JS, Wang CW, Mkumbaye SI, Minja DT, Petersen B, Turner L, Petersen JE, Lusingu JP, Theander TG, Lavstsen T. 2016. Plasmodium falciparum var genes expressed in children with severe malaria encode CIDRalpha1 domains. EMBO Mol Med 8:839–850. https://doi.org/10.15252/emmm.201606188.
24. Mkumbaye SI, Wang CW, Lyimo E, Jespersen JS, Manjurano A, Mosha J, Kavishe RA, Mwakalinga SB, Minja DT, Lusingu JP, Theander TG, Lavstsen T. 2017. The severity of Plasmodium falciparum infection is associated with transcript levels of var genes encoding endothelial protein C receptor-binding P. falciparum erythrocyte membrane protein 1. Infect Immun 85:e00841-16. https://doi.org/10.1128/IAI.00841-16.
25. Lin E, Kiniboro B, Gray L, Dobbie S, Robinson L, Laumaea A, Schopflin S, Stanisic D, Betuela I, Blood-Zikursh M, Siba P, Felger I, Schofield L, Zimmerman P, Mueller I. 2010. Differential patterns of infection and disease with P. falciparum and P. vivax in young Papua New Guinean children. PLoS One 5:e9047. https://doi.org/10.1371/journal.pone.0009047.
26. Mueller I, Schoepflin S, Smith TA, Benton KL, Bretscher MT, Lin E, Kiniboro B, Zimmerman PA, Speed TP, Siba P, Felger I. 2012. Force of infection is key to understanding the epidemiology of Plasmodium falciparum malaria in Papua New Guinean children. Proc Natl Acad Sci U S A 109:10030–10035. https://doi.org/10.1073/pnas.1200841109.
27. Barry AE, Trieu A, Fowkes FJ, Pablo J, Kalantari-Dehaghi M, Jasinskas A, Tan X, Kayala MA, Tavul L, Siba PM, Day KP, Baldi P, Felgner PL, Doolan DL. 2011. The stability and complexity of antibody responses to the major surface antigen of Plasmodium falciparum are associated with age in a malaria endemic area. Mol Cell Proteomics 10:M111.008326. https://doi.org/10.1074/mcp.M111.008326.
28. Cham GK, Turner L, Lusingu J, Vestergaard L, Mmbando BP, Kurtis JD, Jensen AT, Salanti A, Lavstsen T, Theander TG. 2009. Sequential, ordered acquisition of antibodies to Plasmodium falciparum erythrocyte membrane protein 1 domains. J Immunol 183:3356–3363. https://doi.org/10.4049/jimmunol.0901331.
29. Cham GK, Turner L, Kurtis JD, Mutabingwa T, Fried M, Jensen AT, Lavstsen T, Hviid L, Duffy PE, Theander TG. 2010. Hierarchical, domain type-specific acquisition of antibodies to Plasmodium falciparum erythrocyte membrane protein 1 in Tanzanian children. Infect Immun 78:4653–4659. https://doi.org/10.1128/IAI.00593-10.
30. WHO. 2012. Management of severe malaria: a practical handbook. World Health Organization, Geneva, Switzerland.
31. Avril M, Bernabeu M, Benjamin M, Brazier AJ, Smith JD. 2016. Interaction between endothelial protein C receptor and intercellular adhesion molecule 1 to mediate binding of Plasmodium falciparum-infected erythrocytes to endothelial cells. mBio 7:e00615-16. https://doi.org/10.1128/mBio.00615-16.
32. Lennartz F, Bengtsson A, Olsen RW, Joergensen L, Brown A, Remy L, Man P, Forest E, Barfod LK, Adams Y, Higgins MK, Jensen AT. 2015. Mapping the binding site of a cross-reactive Plasmodium falciparum PfEMP1 monoclonal antibody inhibitory of ICAM-1 binding. J Immunol 195:3273–3283. https://doi.org/10.4049/jimmunol.1501404.
33. Stanisic DI, Fowkes FJ, Koinari M, Javati S, Lin E, Kiniboro B, Richards JS, Robinson LJ, Schofield L, Kazura JW, King CL, Zimmerman P, Felger I, Siba PM, Mueller I, Beeson JG. 2015. Acquisition of antibodies against Plasmodium falciparum merozoites and malaria immunity in young children and the influence of age, force of infection, and magnitude of response. Infect Immun 83:646–660. https://doi.org/10.1128/IAI.02398-14.
34. Lusingu JP, Jensen AT, Vestergaard LS, Minja DT, Dalgaard MB, Gesase S, Mmbando BP, Kitua AY, Lemnge MM, Cavanagh D, Hviid L, Theander TG. 2006. Levels of plasma immunoglobulin G with specificity against the cysteine-rich interdomain regions of a semiconserved Plasmodium falciparum erythrocyte membrane protein 1, VAR4, predict protection against malarial anemia and febrile episodes. Infect Immun 74:2867–2875. https://doi.org/10.1128/IAI.74.5.2867-2875.2006.
35. Turner L, Lavstsen T, Mmbando BP, Wang CW, Magistrado PA, Vestergaard LS, Ishengoma DS, Minja DT, Lusingu JP, Theander TG. 2015. IgG antibodies to endothelial protein C receptor-binding cysteine-rich interdomain region domains of Plasmodium falciparum erythrocyte membrane protein 1 are acquired early in life in individuals exposed to malaria. Infect Immun 83:3096–3103. https://doi.org/10.1128/IAI.00271-15.
36. Vigan-Womas I, Guillotte M, Juillerat A, Vallieres C, Lewit-Bentley A, Tall A, Baril L, Bentley GA, Mercereau-Puijalon O. 2011. Allelic diversity of the Plasmodium falciparum erythrocyte membrane protein 1 entails variant-specific red cell surface epitopes. PLoS One 6:e16544. https://doi.org/10.1371/journal.pone.0016544.
37. Hodder AN, Czabotar PE, Uboldi AD, Clarke OB, Lin CS, Healer J, Smith BJ, Cowman AF. 2012. Insights into Duffy binding-like domains through the crystal structure and function of the merozoite surface protein MSPDBL2 from *Plasmodium falciparum*. J Biol Chem 287:32922–32939. https://doi.org/10.1074/jbc.M112.350504.
38. Chiu CY, White MT, Healer J, Thompson JK, Siba PM, Mueller I, Cowman AF, Hansen DS. 2016. Different regions of Plasmodium falciparum erythrocyte binding antigen-175 induce antibody responses to infection of varied efficacy. J Infect Dis 214:96–104. https://doi.org/10.1093/infdis/jiw119.
39. Giraldo J, Vivas NM, Vila E, Badia A. 2002. Assessing the (a)symmetry of concentration-effect curves: empirical versus mechanistic models. Pharmacol Ther 95:21–45. https://doi.org/10.1016/S0163-7258(02)00223-1.
40. Zerbino DR, Birney E. 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res 18:821–829. https://doi.org/10.1101/gr.074492.107.
41. Wernersson R. 2006. Virtual ribosome–a comprehensive DNA translation tool with support for integration of sequence feature annotation. Nucleic Acids Res 34:W385–W388. https://doi.org/10.1093/nar/gkl252.

42. Rask TS, Hansen DA, Theander TG, Gorm Pedersen A, Lavstsen T. 2010. Plasmodium falciparum erythrocyte membrane protein 1 diversity in seven genomes—divide and conquer. PLoS Comput Biol 6:e1000933. https://doi.org/10.1371/journal.pcbi.1000933.
43. Manske M, Miotto O, Campino S, Auburn S, Almagro-Garcia J, Maslen G, O'Brien J, Djimde A, Doumbo O, Zongo I, Ouedraogo JB, Michon P, Mueller I, Siba P, Nzila A, Borrmann S, Kiara SM, Marsh K, Jiang H, Su XZ, Amaratunga C, Fairhurst R, Socheat D, Nosten F, Imwong M, White NJ, Sanders M, Anastasi E, Alcock D, Drury E, Oyola S, Quail MA, Turner DJ, Ruano-Rubio V, Jyothi D, Amenga-Etego L, Hubbart C, Jeffreys A, Rowlands K, Sutherland C, Roper C, Mangano V, Modiano D, Tan JC, Ferdig MT, Amambua-Ngwa A, Conway DJ, Takala-Harrison S, Plowe CV, Rayner JC, Rockett KA, Clark TG, Newbold CI, Berriman M, MacInnis B, Kwiatkowski DP. 2012. Analysis of Plasmodium falciparum diversity in natural infections by deep sequencing. Nature 487:375–379. https://doi.org/10.1038/nature11174.
44. de Castro E, Sigrist CJ, Gattiker A, Bulliard V, Langendijk-Genevaux PS, Gasteiger E, Bairoch A, Hulo N. 2006. ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. Nucleic Acids Res 34:W362–W365. https://doi.org/10.1093/nar/gkl124.
45. Kumar S, Stecher G, Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874. https://doi.org/10.1093/molbev/msw054.
46. Team RC. 2013. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.